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An Iminocoumarin Sulfonamide Based Turn-On Fluorescent Probe for the Detection of Biothiols in Aqueous Solution

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Abstract: A new chemodosimeter for the highly selective sensing and imaging of biothiols was designed and realized in phosphate-buffered saline solution at pH 7.4 through a fluorescence "off–on" response. A unique mechanism featuring a two-step cascade (biothiols→H_2O) sequence for this remarkable recognition is disclosed for the first time.

Introduction

Sensing and signaling of biothiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) is a current research focus owing to the crucial roles of these molecules in maintaining the appropriate redox status of proteins, cells, and organisms.[1] Abnormal levels of biothiols are implicated in a variety of diseases such as cardiovascular disease,[2a] neurotoxicity,[2b] Alzheimer's disease,[2c] and cancer.[2d] The early diagnosis and prevention of these diseases could benefit from the detection of biothiols in living organisms. As a powerful detection tool, reaction-based small-molecule fluorescent probes have been used to sense biothiols because of their high selectivity, sensitivity, versatility, and relatively simple handling.[3] For example, various fluorescent probes based on biothiol addition reactions have been described in recent years,[4] in which the strong nucleophilicity of the mercapto group was utilized. Relatively rare fluorescent probes containing the 2,4-dinitrobenzenesulfonyl (DNBS) moiety have also emerged, and biothiol-mediated cleavage reactions of the corresponding sulfonate ester[5] or sulfonamide[6] were instead employed. In particular, the DNBS moiety is used as an electron-withdrawing group attached to fluorophores for intramolecular charge-transfer (ICT) processes, and it can be removed by cleavage reactions for recovery of the fluorophores. By using this design principle, fluorescent probes for biothiols can achieve high signal-to-noise and turn-on responses.[7] However, most probes bearing the DNBS moiety have worked well only in mixed organic–water media[5b–e,6a,c] or in nearly pure water at high pH values,[6d] and this limits their practical application. Therefore, the development of other DNBS fluorescent probes for biothiols in aqueous buffer solution especially at physiological pH values is still in high demand.

In connection with our continuing research on bifunctional fluorescent probes for relay[8] or divergent recognition[9] of biologically and environmentally important species, herein we report turn-on fluorescent probe **1** based on the iminocoumarin fluorophore tethered to a DNBS moiety for the detection of biothiols in phosphate-buffered saline (PBS)solution at pH 7.4. Notably, the present recognition event demonstrated an unprecedented forward cascade sequence triggered by H_2O as the medium, unlike the usual recycle process.[5,6]

Results and Discussion

Design and Synthesis of DNBS Probe 1

Designed probe **1** was easily synthesized from 10-(1,3-benzothiazol-2-yl)-2,3,6,7-tetrahydro-1*H*,5*H*,11*H*-[1]benzopyrano[6,7,8-*ij*]quinoliz-11-imine (**2**) and 2,4-dinitrobenzene-1-sulfonyl chloride (see the Experimental Section for details). Its structure was determined by ^{1}H NMR and ^{13}C NMR spectroscopy and mass spectrometry (Figures S1–S3, Supporting Information). As shown in Scheme 1, biothiols promote smooth cleavage of the N–S bond within **1** to give intermediate **2**, the hydrolysis[10] of which subsequently occurs to generate coumarin derivative **3**. The whole cascade represents a new pathway of sensing biothiols in aqueous solution.

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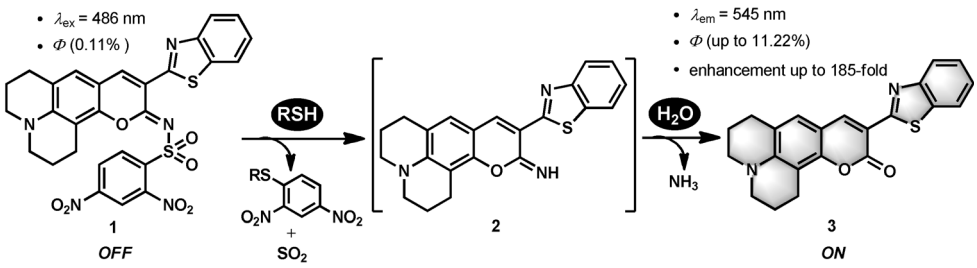


Scheme 1. Tandem mechanism of sensing biothiols.

Fluorescence responses

Considering potential application in bioimaging, the fluorescence of probe **1** was investigated in PBS solution (pH 7.4). As shown in Figure 1, **1** shows a very weak emission band at $\lambda_{em} \approx 524$ nm upon excitation at $\lambda_{ex} = 486$ nm, which is in the range of visible light, and this diminishes damage upon intracellular processes. The weak emission is due to the ICT effect with the iminocoumarin fluorophore as an electron donor and the DNBS unit as an electron acceptor. This behavior was further revealed by time-dependent density functional theory (TD-DFT) calculations (see below, see also Figure 3). Next, Ala, Arg, Asp, Gly, Glu, His, Leu, Ile, Met, Phe, Pro, Ser, Thr, Val, Cys, Hcy, and GSH were used to investigate the selectivity of **1** (20.0 μM) in PBS solution by fluorescence spectroscopy (Figure 1 a). Relative to the fluorescence intensity of other amino acids examined, that of **1** was dramatically increased only in the presence of biothiols (e.g., Cys/Hcy/GSH) with a clear bathochromic shift from 524 to 545 nm. In addition, the fluorescence color changed from almost nonfluorescent to bright green with the addition of these biothiols (Figure 1 d, see also Figure S4). To validate the selectivity of **1** in practice, competition experiments were also measured by the addition of biothiols to the PBS solutions of **1** in the presence of other amino acids (Figure 1 b; see also Figures S5 and S6). Pleasingly, amino acids such as Ala, Arg, Asp, Gly, Glu, His, Leu, Ile, Met, Phe, Pro, Ser, Thr, and Val had no clear interference with the detection of these biothiols. These results suggested that **1** can function as a fluorescent probe for biothiols through a "turn-on" response in PBS solution.

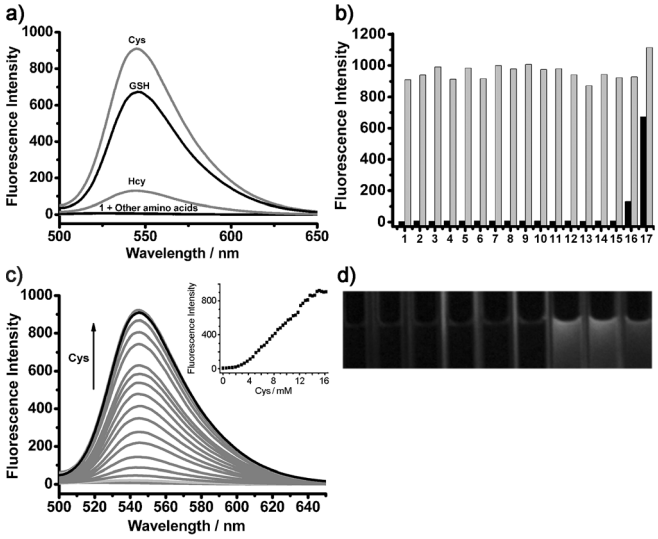


Figure 1. a) Fluorescence spectra of **1** (20.0 μM) with various amino acids (80.0 equiv.) in PBS (pH 7.4) solution ($\lambda_{ex} = 486$ nm). b) The selectivity of **1** (20.0 μM) for Cys. The black bars represent the emission intensity of **1** in the presence of other amino acids (1.6 mM); The gray bars represent the emission intensity that occurs upon the subsequent addition of Cys (1.6 mM) to the above solution. From 1 to 17: none, Ala, Arg, Asp, Gly, Glu, His, Leu, Ile, Met, Phe, Pro, Ser, Thr, Val, Hcy, and GSH ($\lambda_{em} = 545$ nm). c) Fluorescence spectra of **1** (20.0 μM) upon the addition of Cys (0–16.0 mM) ($\lambda_{ex} = 486$ nm) Inset: Fluorescence intensity at $\lambda = 545$ nm as a function of Cys concentration. d) Fluorescence color change of **1**. Left to right: **1** only, Ala, Leu, Phe, Ile, Pro, Lys, GSH, Cys, and Hcy. For a colored version, see the Supporting Information.

The fluorescence titrations of Cys were then conducted by using a 20.0 μM solution of **1** in PBS (pH 7.4) solution (Figure 1 c). Upon the addition of Cys to the solution, a significant increase in the fluorescence emission band centered at $\lambda_{em} = 545$ nm was observed upon excitation at $\lambda_{ex} = 486$ nm. The enhancement in the total fluorescence at $\lambda = 545$ nm was determined to be 185-fold. The fluorescence titrations of Hcy and GSH were also investigated under the same conditions (Figures S7 and S8). The total fluorescence intensity of **1** at $\lambda_{em} = 545$ nm increased 26- and 137-fold for Hcy[11] and GSH in PBS solution, respectively. The fluorescence quantum yields[12] (Φ) of **1** at $\lambda_{em} = 545$ nm increased from 0.11% to 11.22, 1.59, or 8.57% in the presence of Cys, Hcy, or GSH in PBS solution, respectively. The corresponding detection limits[13] were found to be 5.0, 10.0, and 5.0 μM for Cys, Hcy, and GSH, respectively (Figures S9–S11). Kinetic studies of the response of biothiols (1.6 mM) to probe **1** (20.0 μM) in PBS solution at 37 °C were measured (Figures S12–S14). The observed rate constants (k_{obs}) were estimated to be 5.38×10^{-4}, 4.19×10^{-4}, and 4.88×10^{-4} s^{-1} for Cys, Hcy, and GSH, respectively, by fitting the initial fluorescent intensity changes according to a pseudo-first-order kinetics equation. These results indicated that the response of probe **1** to biothiols were not so fast, probably because of the two-step mechanism. In the UV/Vis spectra of **1** in PBS solution, there were almost no changes observed upon the addition of some amino acids, including the biothiols (Figure S15).

Rationalization of the Recognition of Biothiols

To verify the interaction of biothiols with probe **1**, a preparative experiment was performed (see the Supporting Information). Notably, coumarin **3** instead of iminocoumarin **2**[14] was obtained, and it was characterized by ^{1}H NMR and ^{13}C NMR spectroscopy, mass spectrometry, and X-ray diffraction analysis[15] (Figures S16–S18 and Table S1; see also Figure 2). As expected, biothiol-triggered cleavage of the N–S bond of probe **1** to re-

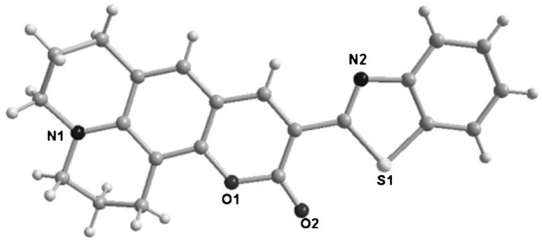


Figure 2. X-ray structure of **3** (crystalline solvent CH_3CN is omitted for clarity).

lease **2**; this species quickly underwent hydrolysis to generate **3** in aqueous solution. Notably, a similar hydrolysis from Nile blue A to Nile red was reported by Lewis very recently.[10] Moreover, **3** was proven to be responsible for the fluorescence emission band at $\lambda_{em} = 545$ nm in PBS solution (Figure S19). Notably, the cascade reaction leading to **3** is distinctively different from simple deprotection of previous DNBS probes.[5,6]

To further understand the relationship between the structural changes of **1**, **3**, and the respective fluorescence response, TD-DFT calculations with the B3LYP/6-31G(d) basis set were performed by using the Gaussian 09 program. As shown in Figure S20, the optimized structure of probe **1** has a dihedral angle of approximately 43.37° between the iminocoumarin ring and the benzothiazole moiety. By contrast, the coumarin and benzothiazole moieties of **3** are essentially planar in the optimized structure, with a dihedral angle of 0.58°, and this results in the formation of delocalized π bonds (Figure S21). Quantum calculations of probe **1** and **3** are summarized in Figure 3 and Table S2. The main contribution transition of probe **1** for the $S_0 \rightarrow S_2$ energy state comes from HOMO→LUMO+1 and HOMO→LUMO+2. As shown in Figure 3, the electron density of the HOMO is mainly localized on the iminocoumarin and benzothiazole moieties, whereas the electron density of LUMO+1 is localized on the iminocoumarin ring and the electron density of LUMO+2 is localized on the DNBS moiety; this is clear indication of a ICT process. As for **3**, the $S_0 \rightarrow S_1$ transition with an oscillator strength (*f*) of 1.0467 is a fully allowed transition, which suggests overlap of the HOMO and LUMO. This result also shows that the $S_1 \rightarrow S_0$ transition is fully allowed,[6c] and therefore, coumarin **3** is potentially fluorescent. Moreover, the electron densities of the HOMO and LUMO of **3** are located over the whole molecule, which is indicative of the strong fluorescence character of **3**. These results are in agreement with turn-on changes observed in the fluorescence spectra of **1** upon the addition of biothiols.

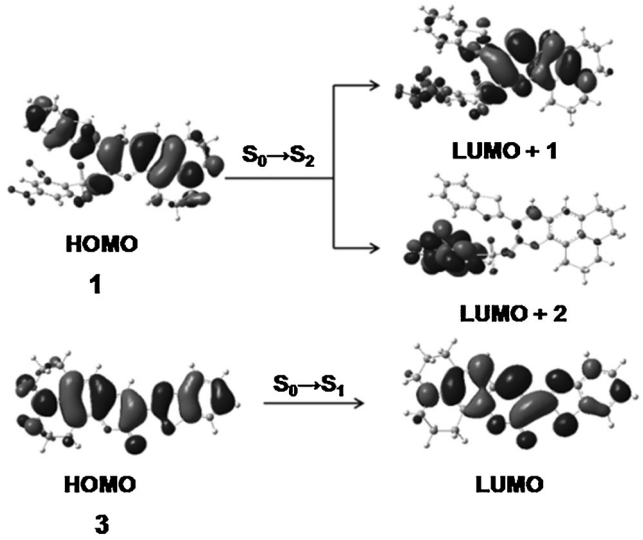


Figure 3. HOMO and LUMO orbital plots of **1** and **3**.

Practical Applications

To explore the practical application of probe **1**, we also evaluated the effect of human blood serum on the fluorescence response of probe **1** to biothiols. As shown in Figure 4, probe **1** exhibited a dramatic turn-on response to human blood serum in PBS (pH 7.4) solution upon excitation at $\lambda = 486$ nm, which indicates that probe **1** is potentially useful for the detection of biothiols in human blood serum.

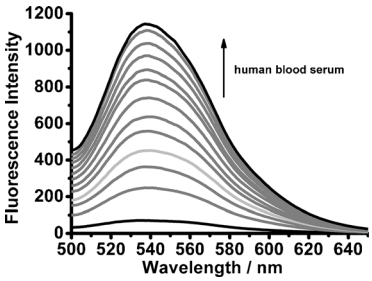


Figure 4. Fluorescence response of **1** with the addition of human blood serum in PBS buffer (pH 7.4) solution ($\lambda_{ex} = 486$ nm).

Finally, the application of probe **1** in the bioimaging of biothiols in living cells was investigated (Figure 5; see also Figure S22). HeLa cells were incubated in PBS (pH 7.4) solution for 1 h at 37°C, and no fluorescence was observed (Figure 5b). If the HeLa cells were incubated with **1** (20.0 μM) in culture

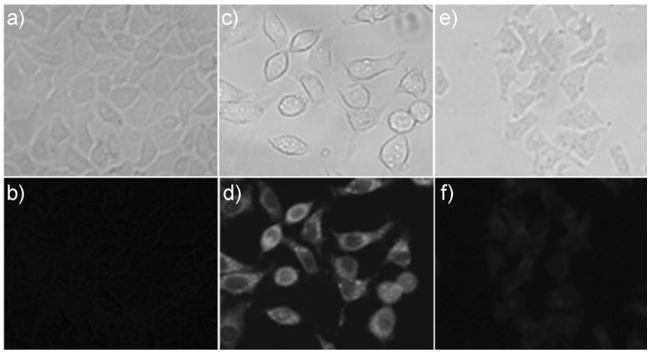


Figure 5. Images of HeLa cells: a) Bright field and b) fluorescence images of HeLa cells. c) Bright field and d) fluorescence images of HeLa cells incubated with **1** (20.0 μM) for 1 h. e) Bright field and f) fluorescence images of HeLa cells pretreated with 500.0 μM of NEM for 0.5 h and then incubated with **1** (20.0 μM) for 1 h. For a color version, see the Supporting Information.

medium for 1 h at 37 °C, strong green fluorescence from the intracellular area was observed (Figure 5 d). In a control experiment, the HeLa cells were pretreated with *N*-ethylmaleimide (NEM, as a thiol-blocking reagent)[16] for 0.5 h and they were further incubated with probe **1** for 1 h, which resulted in very weak fluorescence (Figure 5 f). These results suggest that probe **1** is cell-membrane permeable and also that it works well in living cells.

Conclusions

In summary, we reported chemodosimeter **1** for biothiols on the basis of a novel two-step cascade mechanism: removal of the 2,4-dinitrobenzenesulfonyl moiety promoted by biothiols and facile hydrolysis of the resulting iminocoumarin in phosphate-buffered saline (pH 7.4) solution. Probe **1** displayed almost no fluorescence because of an intramolecular charge-transfer process; upon the addition of biothiols, a significant fluorescence enhancement with a color change from dark to bright green was observed owing to the formation of a strong fluorescent coumarin derivative. The origin of this recognition was further disclosed by time-dependent DFT calculations. Furthermore, this probe was able to detect biothiols in human blood serum and was applied to chemoselective bioimaging in HeLa cells. The present work enriches our continuing investigation into bifunctional fluorescent probes for diverse combinations of analytes.[8,9]

Experimental Section

General methods

Commercially available chemicals were used without further purification. All of the solvents used were analytical-reagent grade. Melting points were determined with a Kofler apparatus. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker 400 MHz or Varian INOVA 600 MHz instrument by using tetramethylsilane as an internal standard. Mass spectra were obtained with a Bruker microTOF mass spectrometer. Fluorescence spectra were determined with a Hitachi F-7000 spectrophotometer. UV/Vis absorption spectra were determined with a Varian UV-Cary100 spectrophotometer. All pH measurements were made with a pH-10C digital pH meter. Inverted microscope fluorescence imaging was performed with a Leica DMI4000B equipped with a charge coupled device camera.

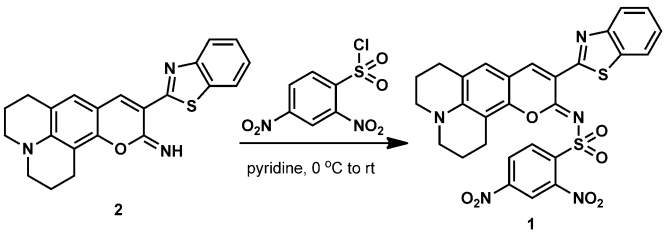
Various stock solutions of the amino acids (100.0 mM) of L-Ala, L-Arg, L-Asp, L-Gly, D-Glu, L-His, L-Leu, L-Ile, L-Met, D-Phe, L-Pro, L-Ser, L-Thr, L-Val, L-Cys, DL-Hcy, and GSH in deionized water were prepared. Stock solution of **1** (2.0 mM) was also prepared in DMF. Test solutions were prepared by placing an aliquot (2.0 μL) of the probe stock solution into a test tube, and it was then diluted to 2.0 mL with deionized water; this was followed by the addition of an appropriate aliquot of each amino acid stock solution. For all measurements, fluorescence spectra were obtained by excitation at $\lambda =$ 486 nm. Both the excitation and emission slit widths were 2.5 nm. Fluorescence quantum yields were determined in solution by using fluorescein ($\Phi = 0.85$ in 0.1 M NaOH) as a standard. For the detection of biothiols in human blood serum, a pure human blood serum sample (2.0 mL) was added to a cuvette, and the fluorescence emission spectrum was recorded per 10 min at room temperature.

The X-ray diffraction data of **3** was obtained with a Bruker SMART Apex CCD area detector diffractometer with a graphite-monochromated MoK_α radiation source ($\lambda = 0.71073$ Å). Lorentz-polarization and absorption corrections were applied for the compound. The structure was solved with direct methods and was refined with full-matrix least-squares on F^2 by using the SHELXL-97 program package.[17] All non-hydrogen atoms were subjected to anisotropic refinement, and all hydrogen atoms were added in idealized positions and were refined isotropically.

The HeLa cells were grown in H-DMEM (Dulbecco's modified Eagle's medium, high glucose) supplemented with 10% FBS (fetal bovine serum) under an atmosphere of 5% CO_2 and 95% air at 37 °C. Cells were seeded in a six-well plate at a density of 10^4 cells per well in culture media. Immediately before the experiments, the cells were washed with PBS buffer, and then cells were treated with 20.0 μM of **1** in culture media for 1 h at 37 °C in a humidified incubator. For the control experiment, the cells were treated with 500.0 μM of *N*-ethylmaleimide (NEM) in culture media for 30 min at 37 °C in a humidified incubator. After washing with PBS, the cells were further incubated with 20.0 μM of **1** in culture media for 1 h.

Synthesis and characterization of probe 1

Iminocoumarin **2** (49.7 mg, 0.13 mmol) was added to dry pyridine (4 mL) under an atmosphere of nitrogen, and the resulting solution was stirred for 30 min at 0 °C. 2,4-Dinitrobenzenesulfonyl chloride (286.1 mg, 1.07 mmol) was then added to the above solution portionwise, and the mixture was stirred for 30 min at 0 °C and for 14 h at room temperature. The solvent was evaporated, and the residue was extracted with ethyl acetate (3×15 mL). The combined organic layer was washed with saturated $NaHCO_3$ (20 mL), water (10 mL), and brine (10 mL), and it was then dried with Na_2SO_4. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography ($CHCl_3$) on silica gel to afford probe **1** (38.1 mg, 47%) as a dark red solid (see Scheme 2). M.p. 258–260 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 9.10$ (s, 1H), 8.90 (s, 1H), 8.65 (d, $J = 8.4$ Hz, 1H), 8.51 (d, $J = 8.8$ Hz, 1H), 8.10 (d, $J = 8.0$ Hz, 1H), 7.99 (d, $J = 8.0$ Hz, 1H), 7.52 (d, $J = 7.2$ Hz, 1H), 7.50 (s, 1H), 7.40 (t, $J = 7.6$ Hz, 1H), 3.41–3.60 (m, 4H), 2.75 (t, $J = 5.6$ Hz, 2H), 2.57 (t, $J = 6.0$ Hz, 2H), 1.90–1.84 ppm (m, 4H); ^{13}C NMR (150 MHz, [D$_6$]DMSO): $\delta = 160.2$, 157.5, 151.8, 150.7, 149.5, 149.4, 147.3, 143.7, 139.3, 135.8, 131.0, 127.7, 127.2, 126.3, 124.6, 122.6, 121.9, 120.0, 109.5, 109.0, 108.8, 105.0, 49.9, 49.3, 29.8, 26.9, 20.1, 19.0 ppm. MS (TOF): *m/z*: 604.0972 $[M+H]^+$.



2 1

Scheme 2. The synthesis of **1**.

Acknowledgements

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[14] For the synthesis and characterization of **2**, see Refs. [9c],[11].

[15] CCDC 1005423 (**3**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

[16] C. R. Yellaturu, M. Bhanoori, I. Neeli, G. N. Rao, *J. Biol. Chem.* **2002**, *277*, 40148 – 40155. The control experiments suggested that NEM cannot react with compound **1** or **3**.

[17] G. M. Sheldrick, *SHELXL-97*, *Program for the Solution of Crystal Structures*, University of Göttingen, Göttingen, Germany, **1997**.

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FULL PAPER

Biosensors

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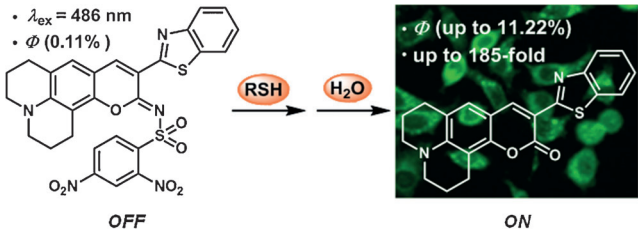
An Iminocoumarin Sulfonamide Based Turn-On Fluorescent Probe for the Detection of Biothiols in Aqueous Solution



The sixth sense: A new chemodosimeter for the selective sensing of biothiols in phosphate-buffered saline solution at pH 7.4 by using a fluorescence "off–on" response is developed. An unprecedented cascade mechanism featuring a two-step cascade (biothiols→H_2O) sequence for this remarkable recognition is noteworthy.